Development of a fish cell culture model to investigate the impact of fish oil replacement on lipid peroxidation

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Last updated February 28, 2011

Abstract Fish oils are rich in omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA), predominantly 20:5n-3 and 22:6n-3, whereas vegetable oils contain abundant C_{18}-PUFA, predominantly 18:3n-3 or 18:2n-6. We hypothesized that replacement of fish oils with vegetable oils would increase the oxidative stability of fish lipids. Here we have used the FHM cell line to test this hypothesis. The FHM cells were readily able to synthesize 20:5n-3 and 24:6n-3 from 18:3n-3 but 22:6n-3 synthesis was negligible. Also, they were readily able to synthesize 20:3n-6 from 18:2n-6 but 20:4n-6 synthesis was negligible. Mitochondrial β-oxidation was greatest for 18:3n-3 and 20:5n-3 and the rates for 16:0, 18:2n-6, 22:6n-3 and 18:1n-9 were significantly lower. Fatty acid incorporation was predominantly into phospholipids (79-97%) with very little incorporation into neutral lipids. Increasing the fatty acid concentration in the growth medium substantially increased the concentrations of 18:3n-3 and 18:2n-6 in the cell phospholipids but this was not the case for 20:5n-3 or 22:6n-3. When they were subjected to oxidative stress, the FHM cells supplemented with either 20:5n-3 or 22:6n-3 (as compared with 18:3n-3 or saturated fatty acids) exhibited significantly higher levels of thiobarbituric reactive substances (TBARS) indicating higher levels of lipid peroxidation. The results are discussed in relation to the effects of fatty acid unsaturation on the oxidative stability of cellular lipids and the implications for sustainable aquaculture.

Keywords Aquaculture, β-oxidation, Cell culture, Fish oil replacement, Lipid peroxidation, Phospholipids, Polyunsaturated fatty acids,

Abbreviations

ALA	α-linolenic acid
ARA	arachidonic acid
BHT	butylated hydroxytoluene
CerPCho	sphingomyelin
DHA	docosahexaenoic acid
EDTA	ethylenediamine tetraacetic acid
EPA	eicosapentaenoic acid
FAF-BSA	fatty acid free-bovine serum albumin
FAME	fatty acid methyl esters
FBS	foetal bovine serum
HP-TLC	high performance-thin layer chromatography
LC-PUFA	long-chain polyunsaturated fatty acids (carbon chain length ≥ C_{20} with ≥ 3 double bonds)
LNA	linoleic acid
NR	neutral red
OLA	oleic acid
PAM	palmitic acid
PBSA	phosphate buffered saline without Ca^{2+} or Mg^{2+}
PCR	polymerase chain reaction
PtdCho	phosphatidylcholine
PtdEtn	phosphatidylethanolamine
Ptd_2Gro	cardiolipin
PtdIns	phosphatidylinositol
PtdOH	phosphatidic acid
PtdSer	phosphatidylserine
PUFA	polyunsaturated fatty acids
SDS	sodium dodecyl sulphate
STA	stearic acid
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TLC	thin-layer chromatography
TN	total neutral lipids
T/V	trypsin/versene

Introduction

Fisheries and aquaculture are major contributors to world food security with ≥15% of animal protein for human consumption being derived from these sources in recent years [1]. Traditionally, feeds for farmed fish have contained high proportions of fish oil derived from wild-catch fisheries. However, in the 10 years to 2007, wild-catch fisheries production has remained static whereas aquaculture production has almost doubled [1]. Thus, there has been considerable interest in the replacement of fish oils with more sustainable oils [2, 3]. The preferred candidates have been vegetable oils from oilseed plants. However, while these are rich in C_{18} polyunsaturated fatty acids (PUFA), they are completely lacking in the omega-3 long-chain PUFA (n-3 LC-PUFA) that are abundant in fish oils. These n-3 LC-PUFA give seafood its reputation as a health food. In particular, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) have been shown to be beneficial in the prevention of cardiovascular disease, rheumatoid arthritis, inflammatory bowel disease, childhood learning and behaviour disorders and adult psychiatric and neurodegenerative illnesses [4].

The fatty acid profile of fish flesh generally reflects the fatty acid profile of their diet [5]. Therefore, the dietary substitution of fish oils with vegetable oils reduces the n-3 LC-PUFA content of fish flesh thereby reducing its human health value [6–10]. In freshwater fish and salmonids (e.g. trout and salmon), this is partially offset by their ability to synthesize LC-PUFA from C_{18} PUFA but in marine fish this is not the case [11–14]. Marine fish appear to be poorly able to synthesize LC-PUFA from C_{18} PUFA and this may reflect the relative abundance of pre-formed LC-PUFA, particularly 22:6n-3, in the marine food web [5]. Although freshwater fish and salmonids have some capacity to synthesize LC-PUFA from C_{18} PUFA, this is insufficient to compensate for a decreased dietary intake of LC-PUFA.

The generally accepted pathway for the synthesis of LC-PUFA from C_{18} PUFA in fish, as in mammals, is a series of desaturation and elongation steps in the endoplasmic reticulum followed by chain shortening in the peroxisomes (Fig. 1). In particular, the synthesis of 22:6n-3 from 18:3n-3 involves a Δ6 desaturation step followed by a chain elongation step, a Δ5 desaturation step and two consecutive chain elongation steps before the chain shortening step. Recent studies indicate that the C_{18} and C_{20} elongation steps are catalysed by an ELOVL5-like elongase whereas the C_{22} elongation step is catalysed by an ELOVL2-like elongase [15, 16]. Studies with fish cell lines indicate that freshwater fish and salmonids have substantial activities of the desaturase and elongase enzymes required to synthesize 20:5n-3 from 18:3n-3 whereas for marine fish there is an apparent limitation at either the C_{18} elongation or the Δ5 desaturation step [11–14]. In contrast, synthesis of 22:6n-3 appears to be limited in all fish species.

Fish lipids are highly susceptible to peroxidative deterioration because of the high proportions of LC-PUFA they contain and this explains the shorter shelf life of fish flesh products as compared with terrestrial animal meat products [17]. The C_{18} PUFA abundant in vegetable oils, 18:2n-6 and 18:3n-3, contain 2 and 3 double bonds, respectively, whereas the LC-PUFA abundant in fish oils, 20:5n-3 and 22:6n-3, contain 5 and 6 double bonds, respectively. Lipid peroxidation occurs when reactive oxygen species (ROS) attack PUFA at their double bonds setting off a chain reaction of hydrogen abstraction and lipid radical formation. This is particularly damaging to cell membranes because of the close proximity of fatty acids in the phospholipid bilayer [17]. Thus, a cell culture model may be useful to predict the effects of fish oil replacement on fish flesh quality, particularly with respect to the oxidative stability of the fish lipids and the integrity of the cellular membranes.

The abundance of individual fatty acids in cellular lipids, particularly phospholipids, is affected by their uptake and esterification into the different lipid classes as well as by their mitochondrial β-oxidation for energy generation and their possible desaturation and/or elongation to form LC-PUFA [5]. Thus, all of these factors need to be considered when developing a cell culture model. Here we have use the FHM cell line derived from fathead minnow (*Pimephales promelas*) to develop this model. In particular we have investigated the capacity of the cell line to synthesize LC-PUFA from C_{18} PUFA, to metabolize various fatty acids by β-oxidation and to incorporate various fatty acids into cellular lipids. Finally, we have investigated the impact of fatty acid unsaturation on the susceptibility of the cells to lipid peroxidation.

Materials and methods

Cells, media and standard culture conditions

A stock culture of the FHM cell line derived from fathead minnow (*Pimephales promelas*) was obtained from the Australian Animal Health Laboratory (CSIRO Livestock Industries, Geelong, Victoria, Australia) and the species of origin was confirmed by polymerase chain reaction (PCR) using the mitochondrial cytochrome oxidase subunit I (*cox1*) primers of Ward et al. [18]. For routine culture, the FHM cells were maintained in 75 cm^2 flasks containing 20 ml Leibovitz's L-15 medium supplemented with antibiotics (100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin) and 10% (v/v) foetal bovine serum (FBS). To subculture or harvest the cells, the medium was decanted, the cell monolayer was rinsed with phosphate buffered saline without Ca^{2+} or Mg^{2+} (PBSA) and the cells were detached with T/V solution containing 0.05% (w/v) trypsin and 0.02% (w/v) ethylenediamine tetraacetic acid (EDTA) in PBSA. For the experiments with [1-^{14}C]-labelled fatty acids, the cells were cultured to 80% confluence in 75 cm^2 flasks containing 20 ml L-15 medium supplemented with 5% (v/v) FBS.

The medium was then replaced with 10 ml of fresh medium containing no FBS and the [1-^{14}C]-labelled fatty acids as complexes with fatty acid free-bovine serum albumin (FAF-BSA). The complexes were prepared as described by Ghioni et al. [19]. For the experiments with unlabelled fatty acids, the cells were cultured to 80% confluence in 75 cm^2 flasks containing 20 ml L-15 medium supplemented with 10% (v/v) FBS and then they were subcultured (at a split ratio of 1 to 12) into 25 cm^2 flasks containing 10 ml L-15 medium supplemented with 2% (v/v) FBS and the unlabelled fatty acids as complexes with FAF-BSA. The complexes were prepared as described by Best et al. [20] with a molar ratio of fatty acid to BSA of 4:1. For all experiments, the culture temperature was 25°C and the incubation period with either the [1-^{14}C]-labelled or the unlabelled fatty acids was 24 h.

LC-PUFA synthesis assay using [1-^{14}C]-labelled fatty acids

The FHM cells were incubated for 24 h in 75 cm^2 flasks containing 1 µCi (2 µM) [1-^{14}C]-labelled 18:2n-6, 18:3n-3 or 20:5n-3 in 10 ml L-15 medium containing no FBS. At the end of the 24 h incubation, the cells were detached with T/V solution and washed with FAF-BSA to remove any residual labelled fatty acid. Total lipid was extracted from the cell pellets by the addition of 5 ml ice-cold chloroform/methanol (2:1, v/v) containing 0.01% (v/v) butylated hydroxytoluene followed by 1 ml 0.88% (w/v) KCl. The method was essentially that of Folch et al. [21] with the modifications described by Tocher et al. [22]. Fatty acid methyl esters (FAME) were prepared by incubating the lipid extract overnight at 50°C with 1 ml of toluene and 2.5 ml of 1% (v/v) H_2SO_4 in methanol. The method was essentially that of Christie [23]. At the end of the incubation, the FAME were extracted by the addition of 2 ml of 2% (w/v) $KHCO_3$ followed by 5 ml isohexane/diethyl ether (1:1, v/v) containing 0.01% (v/v) BHT. The mixture was centrifuged at 500 *g* and the upper layer was re-extracted with 5 ml isohexane/diethyl ether (1:1, v/v) containing no BHT. The upper layers were combined and the solvent was evaporated under a stream of N_2. The dried FAME were dissolved in 100 µl isohexane containing 0.01% (w/v) BHT and separated by thin-layer chromatography (TLC) on 20 x 20 cm TLC plates which had been impregnated with 2 g $AgNO_3$ in 20 ml acetonitrile and dried/activated at 110°C for 30 min. The TLC plates were developed in toluene/acetonitrile (95:5, v/v). The method was essentially as described by Wilson and Sargent [24]. Autoradiography was performed and the areas of silica containing the individual PUFA were scraped into scintillation vials and radioactivity determined using a scintillation counter as previously described [25].

β-oxidation assay using [1-^{14}C]-labelled fatty acids

The FHM cells were incubated for 24 h in 75 cm^2 flasks containing 0.5 µCi (1 µM) [1-^{14}C]-labelled 16:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:5n-3 or 22:6n-3 in 10 ml L-15 medium containing no FBS. At the end of the incubation, 0.5 ml of the medium was removed for scintillation counting and the cells were detached with T/V solution and washed as described above. The washed cell pellets were resuspended in 1 ml PBS and homogenized to disrupt the cells. Acid soluble products were obtained by adding 100 µl of 6% (w/v) FAF-BSA and 1.0 ml ice-cold 4 M $HClO_4$ to 500 µl each of the growth medium and the cell homogenate and then centrifuging to remove the precipitated material. An aliquot of the supernatant (500 µl) was mixed with 4 ml scintillation fluid and radioactivity determined using a scintillation counter as previously described [25].

Incorporation of [1-^{14}C]-labelled fatty acids into various lipid classes

The FHM cells were incubated for 24 h in 75 cm^2 flask containing 1.0 µCi (2 µM) [1-^{14}C]-labelled 16:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:5n-3 or 22:6n-3 in 10 ml L-15 medium containing no FBS. At the end of the 24 h incubation, the cells were harvested and total lipid was extracted as described above. To separate the various lipid classes, the total lipid was dissolved in 100 µl chloroform/methanol (2:1, v/v) and applied to a high performance-thin layer chromatography (HPTLC) plate. The plate was developed in methyl acetate/isopropanol/chloroform/methanol/0.25% (w/v) aqueous KCl (25/25/25/10/9, by volume) as described by Vitiello and Zanetta [26]. The separated lipids were stained with iodine and the corresponding bands of silica were scraped into scintillation vials. Scintillation fluid (2.5 ml) was added and radioactivity determined using a scintillation counter as above.

Incorporation of unlabelled fatty acids into the FHM cell phospholipids

The FHM cells were incubated for 24 h in 25 cm^2 flasks containing 10 ml L-15 medium supplemented with 2% (v/v) FBS and various concentrations of unlabelled 18:3n-3, 18:2n-6, 20:5n-3, 20:4n-6 or 22:6n-3. At the end of the incubation, the cells were harvested and total lipid was extracted from approximately 10^6 cells according to the method of Folch et al. [21]. The phospholipids were separated from the neutral lipids by TLC with petroleum ether/glacial acetic acid (3:1, v/v) and then they were transmethylated by incubation with 1% (v/v) H_2SO_4 in methanol for 3 h at 70°C. The resulting FAME were extracted in heptane and analyzed by gas chromatography using a Hewlett-Packard 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) fitted with a flame ionisation detector and a BPX-70 50 m capillary column coated with 70% (v/v) cyanopropyl

polysilphenylene-siloxane (0.25 mm film thickness and 0.32 mm internal diameter, SGE, Australia). The carrier gas was helium at a flow rate of 2.0 mL min^{-1} and the split-ratio was 20:1. The injection port temperature was 250°C and the detector temperature was 300°C. The column temperature was increased from 140°C to 220°C at a rate of 4°C min^{-1} and then held at 220°C for up to 3 min. The identity of each fatty acid peak in the chromatogram was ascertained by comparison with an authentic lipid standard (Nu-Chek Prep, Inc., MN, USA). The amount of each fatty acid was quantified by comparing its peak area with the peak area of a heptadecaenoic acid (17:0) internal standard added prior to lipid extraction.

Effect of fatty acid unsaturation on cell viability and lipid peroxidation

The FHM cells were seeded into 6-well plates at a density of 5 x 10^5 cells per well in L-15 medium containing 2% (v/v) FBS. The cells were allowed to attach overnight and then they were incubated for 24 h with fresh medium containing 2% (v/v) FBS and 20 µM 16:0, 18:0, 18:3n-3, 20:5n-3 or 22:6n-3 coupled with FAF-BSA in a 4:1 fatty acid to BSA ratio. At the end of the incubation period, the cell monolayer was rinsed twice with PBSA and then lipid peroxidation was induced by treating the cells for 1 h with 500 µM cumene hydroperoxide plus 100 nM hemin in PBSA. Following this treatment, the cell monolayer was rinsed twice with PBSA and the cells were subjected to either the Neutral Red (NR) cell viability assay [27] or the thiobarbituric acid reactive substances (TBARS) assay for lipid peroxidation [28]. Briefly, the NR cell viability assay involved incubating the cells for 3 h in NR dye diluted in L-15 containing 10% (v/v) FBS and then fixing them in 10% (w/v) $CaCl_2$:4% (v/v) formaldehyde before solubilising the incorporated dye with 50% (v/v) ethanol:1% (v/v) acetic acid and reading the absorbance at 550 nm. Viable cells take up the dye whereas non-viable cells do not. For the TBARS assay, the cells were lysed with 1% (w/v) sodium dodecyl sulphate (SDS), the cell lysates were mixed 1:1 with the TBARS reagent and the mixture was incubated for 2 h at 75°C before removing any insoluble material by centrifugation and reading the fluorescence using excitation/emission wavelengths of 540/590 nm. The TBARS reagent contained 25 mM thiobarbituric acid (TBA), 15% (w/v) trichloroacetic acid and 0.005% (w/v) BHT dissolved in 1 M HCl.

Statistical analyses

Statistical analyses were performed using SPSS software. The Levene statistic was calculated to test for homogeneity of variances and means were compared using one-way ANOVA followed by Tukey's *post hoc* test. Differences where the P value was >0.05 were considered to be significant.

Results

Effect of the FBS concentration in the growth medium on the fatty acid composition of the FHM cell phospholipids

The fatty acid composition of the FHM cell phospholipids closely resembled that of the FBS in the growth medium, with the exceptions that 16:0 and 18:0 were less abundant and 18:1n-9 was more abundant (Table 1). Increasing the concentration of FBS in the growth medium from 2 to 20% (v/v) significantly increased the concentrations of 22:5n-3, 22:6n-3 and 20:4n-6 in the cell phospholipids (Fig. 2).

Metabolism of [1-^{14}C]-labelled fatty acids by the FHM cells

Radioactivity from [1-^{14}C]18:3n-3 was readily recovered in 20:4n-3, 20:5n-3, 22:5n-3 and 24:6n-3 but there was relatively little recovery in 22:6n-3 (Table 2). Thus, the cells expressed considerable desaturase and elongase activity but only limited peroxisomal chain shortening activity. This was confirmed by supplying the cells with [1-^{14}C]20:5n-3. A somewhat different pattern was observed when the cells were supplied with [1-^{14}C]18:2n-6. In this case, the label was incorporated predominantly into 20:3n-6. This indicated considerable Δ6 desaturase activity to yield 18:3n-6 followed by elongase activity to yield 20:3n-6 but limited Δ5 desaturase activity to yield 20:4n-6. Thus, the FHM Δ5 desaturase apparently preferred the n-3 over the n-6 substrate.

The capacity of the FHM cells to metabolise fatty acids by mitochondrial β-oxidation was also investigated (Fig. 3). This showed that 18:3n-3 and 20:5n-3 were more readily oxidized than 16:0, 18:2n-6 or 22:6n-3 which in turn were more readily oxidized than 18:1n-9. This result was consistent with the high retention levels for 18:1n-9 in the cell phospholipids (Table 1).

Incorporation of [1-^{14}C]-labelled fatty acids into lipid classes in the FHM cells

Total incorporation into all lipid classes was similar for 18:1n-9, 18:2n-6 and 20:5n-3 but significantly greater for 18:3n-3 and significantly less for 16:0 and 22:6n-3 (Table 3). The lower incorporation rate for 16:0 was consistent with its lower steady state level in the FHM cell phospholipids as compared with FBS (Table 1). This contrasts with the higher steady state level for 18:1n-9 which was apparently due to a lower rate of β-oxidation. For all of the fatty acids tested, the majority of the label (79-97%) was incorporated into phospholipids with very little incorporation into neutral lipids. For 16:0, 18:1n-9 and 18:2n-6, most of the label (44-50%) was incorporated into phosphatidylcholine (PtdCho)

with phosphatidylethanolamine (PtdEtn) ranked second. For 18:3n-3 and 20:5n-3, the ranking was reversed with the greatest incorporation into PtdEtn followed by PtdCho. For 22:6n-3, the pattern was different again with the greatest incorporation into PtdEtn followed by phosphatidylserine (PtdSer) and then PtdCho. Thus, the lower overall incorporation of 22:6n-3 into total lipids was associated with lower incorporation into PtdCho and higher incorporation into PtdSer. In addition, as the unsaturation of the fatty acids increased, there was a trend away from incorporation into PtdCho.

Effect of concentration on the accumulation of unlabelled fatty acids in the FHM cell phospholipids

Fig. 4 shows the effects of increasing concentrations of 18:3n-3 or 18:2n-6 in the growth medium on their accumulation in the cell phospholipids. In both cases, accumulation in the cell phospholipids paralleled the increase in concentration in the growth medium with an almost linear relationship at low concentrations up to 5 µM and a tendency towards saturation at higher concentrations up to 20 µM. The accumulation of 18:3n-3 was roughly paralleled by an accumulation of 20:3n-3, the direct elongation product of 18:3n-3. Thus, Δ6 desaturase activity, which would have produced 18:4n-3, appeared to be limiting. In contrast, 18:2n-6 was a poor substrate for direct elongation as evidenced by limited accumulation of 20:2n-6. However, 20:3n-6 accumulation was significant and this is consistent with the earlier observation of limited Δ5 desaturase activity towards 20:3n-6 in the [1-^{14}C]-labelling experiment (Table 2).

Further metabolism of 18:3n-3 to produce 20:5n-3 and 22:6n-3 or of 18:2n-6 to produce 20:4n-6 was negligible. This was surprising given the results of the [1-^{14}C]-labelling experiment which showed significant synthesis of 20:5n-3 from 18:3n-3 (Table 2). To determine whether this apparent anomaly was due to limited incorporation of higher concentrations of LC-PUFA into the cellular phospholipids, the FHM cells were supplied with various concentrations of either 20:5n-3, 20:4n-6 or 22:6n-3 (Figs. 5 and 6). Incorporation of 20:5n-3 into the cell phospholipids increased approximately linearly with the increase in the concentration in the growth medium up to a concentration of 5 µM whereas at higher concentrations it reached a plateau. The accumulation of 20:5n-3 was paralleled by an accumulation of 22:5n-3, the direct elongation product of 20:5n-3. Again this indicated significant elongase activity but limited desaturase activity towards n-3 PUFA in the FHM cells. Further metabolism of 20:5n-3 to produce 22:6n-3 by chain shortening was negligible.

Incorporation of 20:4n-6 was less straightforward (Fig. 5). At lower concentrations up to 5 µM, there was some evidence of increasing incorporation with increasing concentration in the medium. However, at higher concentrations in the medium (5 to 20 µM) there was no increase in incorporation of 20:4n-6 into the cells. This was not unexpected given that the cells were cultured in the presence of

2% (v/v) FBS which is relatively rich in 20:4n-6 and therefore the capacity of the cell phospholipids to incorporate 20:4n-6 may already have been saturated.

Incorporation of 22:6n-3 into the cell phospholipids saturated at a very low concentration of only 2 µM (Fig. 6). This was lower than the concentration of approximately 5 µM observed for 20:5n-3. This was consistent with the ^{14}C-labelling data which showed significantly lower incorporation of 22:6n-3 into total lipid (Table 3). Interestingly, there was also some evidence for retro-conversion of 22:6n-3 to 20:5n-3 (Fig. 6).

Effect of fatty acid unsaturation on cell viability and lipid peroxidation

There was no significant difference in cell viability between any of the fatty acid treatments except for the treatment with 18:0 which showed significantly higher cell viability (Fig. 7). For TBARS, the concentration was significantly greater in the cells supplemented with either 20:5n-3 or 22:6n-3 as compared with the cells supplemented with either 16:0, 18:0 or 18:3n-3. Thus, increasing fatty acid unsaturation increased the susceptibility of the cells to lipid peroxidation but did not decrease cell viability.

Discussion

The aim of this study was to develop a cell culture model for the impact of fish oil replacement with vegetable oils on the oxidative stability of fish lipids. Fish oils are rich in LC-PUFA with 5 or 6 double bonds whereas vegetable oils are rich in C_{18} PUFA with only 2 or 3 double bonds [3] and since the fatty acid composition of fish lipids reflects the fatty acid composition of their diet and fatty acid susceptibility to peroxidation increases with increasing unsaturation, we hypothesized that replacing LC-PUFA with C_{18} PUFA would increase the oxidative stability of fish lipids. Previous studies had shown that fish cells in culture have decreased proportions of n-3 LC-PUFA in their lipids as compared with whole fish or fish fillets and this was attributed to the low concentrations of n-3 LC-PUFA in the mammalian serum which is the usual source of fatty acids for cells in culture [13, 22, 29]. The present study confirmed this and in particular we found that the proportions of the beneficial n-3 LC-PUFA, 20:5n-3 and 22:6n-3, were much lower in the FHM cells in culture than in fish tissues in general.

The effects of fish oil replacement on the fatty acid profile of fish flesh are determined by fatty acid metabolism and incorporation into cellular lipids. Previous studies had shown that cell lines derived from fresh water or anadromous fish species (Atlantic salmon, rainbow trout and common carp) were

353 readily able to synthesize 20:5n-3 from 18:3n-3 whereas cell lines derived from marine fish species
354 (turbot and gilthead sea bream) were not [11–14]. In turbot this was attributed to low C_{18} to C_{20}
355 elongase activity whereas in gilthead sea bream it was attributed to low Δ5 desaturase activity.
356 Importantly, none of these cell lines could synthesize significant quantities of 22:6n-3. In the present
357 study, the FHM cell line was readily able to synthesize 20:5n-3, 22:5n-3 and 24:6n-3 from 18:3n-3
358 and/or 20:5n-3 but 22:6n-3 synthesis was negligible. Thus, the FHM cell line expressed substantial Δ5
359 and Δ6 desaturase activities as well as $C_{18\text{-}20}$, $C_{20\text{-}22}$ and $C_{22\text{-}24}$ elongase activities but negligible
360 peroxisomal chain shortening activity. This was similar to cell lines from other freshwater fish
361 species.
362
363 The FHM cells were also readily able to desaturate 18:2n-6 to 18:3n-6 and further elongate this to
364 20:3n-6 but they synthesized little 20:4n-6. This suggested limited Δ5 desaturase activity towards n-6
365 fatty acids, which was in contrast to the results obtained with the n-3 substrates. Thus, the Δ5
366 desaturase of the FHM cell line appeared to prefer n-3 over n-6 fatty acids. Previous studies with cell
367 lines from Atlantic salmon, rainbow trout, turbot and gilthead sea bream also showed limited Δ5
368 desaturase activity towards 20:3n-6 [11–13]. Thus, this appears to be a general phenomenon for fish
369 cell lines regardless of whether they originate from freshwater, anadromous or marine fish species.
370
371 Fatty acid utilization via β-oxidation in the FHM cells was investigated by supplying them with
372 [1-^{14}C]-labelled 16:0, 18:1n-9 18:2n-6, 18:3n-3, 20:5n-3 or 22:6n-3. The β-oxidation rates were
373 similar for 16:0, 18:2n-6 and 22:6n-3 but significantly lower for 18:1n-9 and significantly higher for
374 18:3n-3 and 20:5n-3. In a previous study with Atlantic salmon hepatocytes isolated from fish fed a
375 fish oil-based diet, β-oxidation rates were similar for 16:0 and 18:2n-6, slightly lower for 18:1n-9 and
376 much lower for 18:3n-3, 20:5n-3 and 22:6n-3 [30]. Feeding the fish a predominantly vegetable oil-
377 based diet significantly increased the β-oxidation of 18:1n-9, 18:2n-6, 18:3n-3 and 20:5n-3 but the
378 rank order was still similar. Clearly the metabolism of primary hepatocytes is likely to be different to
379 that of cells maintained in culture for long periods of time and therefore more work needs to be done
380 to understand β-oxidation in fish cell lines. In particular, the rates measured using radiolabelled
381 substrates may be affected by the pool sizes of unlabelled substrates in the cells due to dilution of the
382 label. The FHM cell phospholipids were particularly rich in 16:0 and 18:1n-9, with moderate levels of
383 18:2n-6 and 22:6n-3 and low levels of 18:3n-3 and 20:5n-3. Thus, the β-oxidation rates for 16:0 and
384 18:1n-9, in particular, may have been underestimated. In addition, the incorporation of 22:6n-3 into
385 total cellular lipids was significantly lower compared with the other fatty acids and this may have
386 resulted in an underestimation of the β-oxidation rate for this fatty acid as well. Overall, therefore, the
387 FHM cells were readily able to oxidize all six of the different fatty acids supplied to them.

The incorporation of fatty acids into cellular lipids in the FHM cells was studied in two different ways, firstly using a trace (2 μM) concentration of [1-^{14}C]-labelled fatty acids and secondly using increasing concentrations of unlabelled fatty acids in the range 1-20 μM. Previous studies with fish cell lines and trace concentrations of [1-^{14}C]-labelled 18:2n-6, 18:3n-3 and 20:5n-3 had shown that the label was incorporated predominantly (mostly >90%) into phospholipids, especially PtdCho and PtdEtn, with very little incorporation into neutral lipids [11, 13, 14]. The FHM cell line behaved the same way. In addition, we found that [1-^{14}C]-labelled 16:0, 18:1n-9 and 22:6n-3 were also incorporated predominantly into phospholipids. Interestingly, there was substantial incorporation of [1-^{14}C]-labelled 22:6n-3 into PtdSer and less incorporation into PtdCho than for the other fatty acids. This has also been observed for cell lines from rainbow trout, Atlantic salmon and turbot [31–33]. PtdSer is less abundant than PtdCho in fish cell lines in general [13, 14] and this may explain the lower level of incorporation of [1-^{14}C]-labelled 22:6n-3 into total lipid observed in the present study.

Increasing the concentration of unlabelled 18:3n-3 in the culture medium from 1 to 20 μM resulted in a roughly corresponding increase in the concentration of this fatty acid in the FHM cell phospholipids but there was no corresponding increase in the conventional desaturation/elongation products 20:5n-3, 22:5n-3 or 24:6n-3. Instead, there was an accumulation of 20:3n-3. This fatty acid is produced by the direct elongation of 18:3n-3. Thus, Δ6 desaturase activity apparently limited the flux through the conventional pathway as the 18:3n-3 concentration was increased above the trace concentration used in the [1-^{14}C]-labelling experiments. This was in contrast to the results of previous studies with rainbow trout and Atlantic salmon cell lines supplied increasing concentrations of unlabelled fatty acids [34, 35]. In those studies, increasing the concentration of unlabelled 18:3n-3 from 5 to 50 μM resulted in a greater increase in 20:5n-3 than 20:3n-3. The main difference between the present and the previous studies is that the previous studies analyzed total lipid whereas in the present study we analyzed phospholipids. Thus, there may have been incorporation into neutral lipids which was not detected in the present study. Tocher et al. [34] noted that there were no large increases in neutral lipids in either rainbow trout or turbot cell lines supplied fatty acids at concentrations up to 20 μM however lipid droplets did appear when the concentration was increased above 50 μM. Thus, in the future, it will be interesting to investigate the incorporation of fatty acids into neutral lipids in the FHM cells and compare this with their incorporation into phospholipids to determine the relative distributions as fatty acid concentrations are increased.

Incorporation of 18:2n-6 into the FHM cell phospholipids with increasing concentrations of this fatty acid in the growth medium showed a similar pattern to that observed with 18:3n-3 except that 20:3n-6 was the major desaturation/elongation product incorporated. This is produced by the conventional Δ6 desaturation of 18:2n-6 to yield 18:3n-6 followed by the conventional elongation of 18:3n-6 to yield

20:3n-6. Thus, the result obtained with unlabelled 18:2n-6 was similar to that obtained with labelled 18:2n-6 and this confirmed that Δ5 desaturase was the limiting step in n-6 LC-PUFA synthesis in the FHM cell line. This was consistent with the results of previous work with other freshwater and anadromous fish cell lines [34, 35]. Taking the results with 18:3n-3 and 18:2n-6 together, it suggests that fish cells in culture are suitable for the study of the effects of increasing concentrations of C_{18} PUFA derived from vegetable oils. However, in order to have a control representative of a fish oil-based diet, it will be necessary to supply the cultured cells with either 20:5n-3 or 22:6n-3 to significantly raise the levels of these fatty acids in the cellular lipids. Thus, this was also investigated.

When the FHM cells were supplied with increasing concentrations of 20:5n-3, there was a corresponding increase in the concentration of this fatty acid in their phospholipids but only up to a culture medium concentration of 10 μM. Above this concentration, the incorporation of 20:5n-3 into the cell phospholipids did not change significantly. Thus, incorporation of 20:5n-3 saturated out at a lower concentration than incorporation of either of the C_{18} fatty acids. This was in contrast to the results obtained with a cell line from turbot, a marine fish species [34]. In that case 20:5n-3 incorporation continued increasing up to a culture medium concentration of at least 50 μM. Perhaps this reflects a greater capacity for incorporation of 20:5n-3 in marine fish than in freshwater fish but it should also be noted that total lipid was analyzed in the previous study whereas only phospholipids were analyzed in the present study. The direct incorporation of 22:6n-3 into the FHM cell phospholipids was also investigated. This saturated at a very low concentration of only 2 μM in the culture medium. This has not been studied before and it clearly warrants further investigation.

In general fish flesh products (i.e. seafood) have a shorter shelf life than terrestrial animal meat products and this is due to the greater susceptibility of fish flesh to lipid peroxidation as a result of the higher proportions of LC-PUFA in fish lipids [17]. Thus, it can be hypothesized that feeding farmed fish vegetable oils with their abundance of either 18:3n-3 or 18:2n-6 as opposed to fish oils with their abundance of 20:5n-3 and 22:6n-3 will result in extended shelf life. To test this hypothesis in our *in vitro* system, we enriched the FHM cells with either saturated fatty acids (16:0 or 18:0), PUFA (18:3n-3) or LC-PUFA (20:5n-3 or 22:6n-3) and then subjected them to oxidative stress to stimulate lipid peroxidation. The cells enriched with 20:5n-3 or 22:6n-3 had significantly higher concentrations of TBARS than the cells enriched with 16:0, 18:0 or 18:3n-3. TBARS is a commonly used indicator of lipid peroxidation in fish [17]. Thus, increasing the unsaturation of the FHM lipids increased the susceptibility of the cells to lipid peroxidation. Therefore in this respect, the cells are a good model for the effects of dietary fish oil replacement on the flesh quality of farmed fish. Previous studies with Atlantic salmon fed diets containing either fish oil, vegetable oil or oils enriched with either 20:5n-3 or 22:6n-3 showed that increasing dietary unsaturation increased indicators of oxidative stress and

apoptosis in liver and white adipose tissue [36, 37]. In particular, fish fed diets enriched with either 20:5n-3 or 22:6n-3 had greater activities of the antioxidant enzyme superoxide dismutase and the apoptosis marker caspase 3 as well as reduced integrity of their mitochondria as evidenced by almost undetectable levels of β-oxidation of ^{14}C-palmitoyl CoA. More direct evidence for the effect of dietary fatty acid unsaturation on the oxidative stability of fish lipids comes from a study with grass carp fed diets containing either lard, plant oil or fish oil [38]. In that study, TBARS concentration in the plasma of the fish increased with increasing dietary lipid content and also with increasing unsaturation of the dietary oil. It has also been shown that supplementing the growth medium with the antioxidant α-tocopherol (vitamin E) decreased the longer term inhibitory effect of 20:5n-3 supplementation on the growth of a turbot cell line [39].

In conclusion, the FHM cells had substantial capacity to synthesize 20:5n-3 from 18:3n-3 but only limited capacity to synthesize 22:6n-3. In addition they had substantial capacity to synthesize 20:3n-6 from 18:2n-6 but only limited capacity to synthesize 20:4n-6. Together the data indicated limited chain shortening activity in the peroxisomes and limited Δ5 desaturase activity especially towards 20:3n-6 but also towards 20:4n-3. At a trace (2 μM) concentration, fatty acid incorporation into total lipid was greatest for 18:3n-3 followed closely by 18:2n-6 and 20:5n-3 with incorporation of 22:6n-3 being substantially less. Fatty acid incorporation into phospholipids saturated out at much lower concentrations for 20:5n-3 and 22:6n-3 than for 18:3n-3 and 18:2n-6 indicating that the cell membranes had limited capacity for incorporation of 20:5n-3 and 22:6n-3 presumably because of the need to maintain appropriate levels of fluidity. Thus, dietary fish oil replacement with vegetable oils may result in substantial displacement of LC-PUFA by C_{18} PUFA in fish cell membranes. Despite the limited capacity of the cell membranes to incorporate either 20:5n-3 or 22:6n-3, the FHM cells still showed greater susceptibility to lipid peroxidation when the culture medium was supplemented with LC-PUFA as compared with either 18:3n-3 or saturated fatty acids. Thus, the FHM cell line can be a useful model for the effects of dietary fish oil replacement on lipid stability and shelf life in farmed fish.

Acknowledgements MKG was the recipient of a Flinders University Postgraduate Research Scholarship and KAS received funding from the Flinders University Outside Studies Program.

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598

599 Figure legends

600

601 **Fig. 1** The generally accepted pathway for the synthesis n-3 and n-6 LC-PUFA from their C_{18} fatty
602 acid precursors in fish (adapted from Miller et al., 2008) showing the alternative reactions when Δ6
603 fatty acyl desaturase activity is limiting (broken lines). Abbreviations: Δ5, Δ5 fatty acyl desaturase;
604 Δ6, Δ6 fatty acyl desaturase; ARA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic
605 acid; ELOVL2, fatty acyl elongase 2; ELOVL5, fatty acyl elongase 5; EPA, eicosapentaenoic acid;
606 LNA, linoleic acid; short, peroxisomal shortening of LC-PUFA.

607

608 **Fig. 2** The effect of increasing FBS concentration in the growth medium on the PUFA content of the
609 FHM cell phospholipids. The growth medium contained 2% (v/v) BSA, 2 μM 18:3n-3 and increasing
610 concentrations of FBS and the cells were cultured for 24 h at 25°C. Each data point represents the
611 mean of 3 flasks and the vertical bars represent the standard error of the mean. For each fatty acid,
612 data points with different letters represent values that are significantly different from one another at
613 the P = 0.05 level.

614

615 **Fig. 3** β-oxidation activity of the FHM cell line with various fatty acids. The cells were incubated for
616 24 h at 25°C with 1 μCi (2 μM) of the [1-^{14}C]-labelled fatty acids in L-15 medium containing no FBS.
617 Data points with different letters represent values that are significantly different from one another at
618 the P = 0.05 level.

619

620 **Fig. 4** The effect of increasing 18:3n-3 or 18:2n-6 concentration in the growth medium on the PUFA
621 content of the FHM cell phospholipids. The growth medium contained 2% (v/v) BSA and increasing
622 concentrations of either 18:3n-3 or 18:2n-6 and the cells were cultured for 24 h at 25°C. Each data
623 point represents the mean of 3 flasks and the vertical bars represent the standard error of the mean.
624 For each fatty acid, data points with different letters represent values that are significantly different
625 from one another at the P = 0.05 level.

626

627 **Fig. 5** The effect of increasing 20:5n-3 or 20:4n-6 concentration in the growth medium on the PUFA
628 content of the FHM cell phospholipids. The growth medium contained 2% (v/v) BSA and increasing
629 concentrations of either 20:5n-3 or 20:4n-6 and the cells were cultured for 24 h at 25°C. Each data
630 point represents the mean of 3 flasks and the vertical bars represent the standard error of the mean.
631 For each fatty acid, data points with different letters represent values that are significantly different
632 from one another at the P = 0.05 level.

633

634 **Fig. 6** The effect of increasing 22:6n-3 concentration in the growth medium on the PUFA content of
635 the FHM cell phospholipids. The growth medium contained 2% (v/v) BSA and increasing
636 concentrations of 22:6n-3 and the cells were cultured for 24 h at 25°C. Each data point represents the
637 mean of 3 flasks and the vertical bars represent the standard error of the mean. For each fatty acid,
638 data points with different letters represent values that are significantly different from one another at
639 the P = 0.05 level.
640
641 **Fig. 7** The effect of increasing fatty acid unsaturation on cell viability and lipid peroxidation in the
642 FHM cells. The cells were supplied with the various fatty acids at a concentration of 20 µM in L-15
643 medium containing 2% (v/v) FBS. The control contained ethanol at the concentration present in the
644 fatty acid supplements. Three separate flasks of cells were analyzed for each data point and the
645 columns represent the mean while the vertical bars represent the standard deviation. For the TBARS
646 assay, * indicates a significant difference (at the P = 0.05 level) between the results for 20:5n-3 and
647 22:6n-3 and the results for the other fatty acids. For the NR assay * indicates a significant difference
648 (at the P = 0.05 level) between the results for 18:0 and the results for the other fatty acids.

649 **Table 1** Fatty acid composition of foetal bovine serum (FBS) and the phospholipids of FHM cells
650 maintained in L-15 medium containing 10% (v/v) FBS.

Fatty acid	Fatty acid composition (% of total fatty acids)	
	FBS	FHM cells in L-15 + 10% FBS
14:0	0.8	1.3
16:0 (PAM)	23.0	17.8
18:0 (STA)	18.8	9.0
Total saturates[a]	45.3	32.0
16:1n-9	0.9	2.0
16:1n-7	1.3	2.2
18:1n-9 (OLA)	15.3	24.1
18:1n-7	2.0	7.9
Total monounsaturates[b]	23.5	42.4
18:2n-6 (LNA)	7.6	1.8
18:3n-6	0.3	0.4
20:2n-6	0.2	0.4
20:3n-6	3.4	3.0
20:4n-6 (ARA)	9.9	8.4
Total n-6 PUFA[c]	23.3	15.3
18:3n-3 (ALA)	0	0.2
20:5n-3 (EPA)	0.3	0.2
22:5n-3 (DPA)	3.7	1.5
22:6n-3 (DHA)	4.0	2.9
Total n-3 PUFA[d]	8.1	4.9
Total PUFA	31.4	20.2
n-3/n-6	0.35	0.32

651 Data are expressed as % of total fatty acids. For FBS, the data are the mean of 3 separate batches. For
652 the FHM cells, the data are the mean of 3 replicate flasks; [a]also includes 15:0, 17:0, 20:0, 22:0 and
653 24:0; [b]also includes 20:1, 22:1 and 24:1; [c]also includes 22:4n-6; [d]also includes 18:4n-3 and 20:4n-3.
654 Abbreviations: palmitic acid, PAM; stearic acid, STA; oleic acid, OLA; linoleic acid, LNA;
655 arachidonic acid, ARA; α-linolenic acid, ALA; eicosapentaenoic acid, EPA; docosahexaenoic acid,
656 DHA.

657 **Table 2** Metabolism of [1-^{14}C]-labelled 18:3n-3, 20:5n-3 and 18:2n-6 by
658 desaturation and elongation in FHM cells

Fatty acid	Substrate		
	[1-^{14}C]18:3n-3	[1-^{14}C]20:5n-3	[1-^{14}C]18:2n-6
18:3n-3	14.5 ± 0.7	-	-
20:3n-3	7.7 ± 0.3	-	-
18:4n-3/24:5n-3	9.9 ± 0.5	-	-
20:4n-3	18.3 ± 0.3	-	-
22:4n-3	1.9 ± 0.2	-	-
20:5n-3	22.1 ± 0.8	42.7 ± 1.6	-
22:5n-3	15.2 ± 0.5	26.9 ± 0.7	-
24:5n-3	-[a]	8.3 ± 1.3	
22:6n-3	1.0 ± 0.1	3.2 ± 0.7	-
24:6n-3	9.4 ± 0.6	18.9 ± 0.5	
18:2n-6	-	-	53.4 ± 1.4
20:2n-6	-	-	6.7 ± 0.3
18:3n-6	-	-	7.1 ± 1.0
20:3n-6	-	-	26.8 ± 1.0
22:3n-6	-	-	1.6 ± 0.2
20:4n-6	-	-	4.5 ± 0.3

659 [a]indicates not applicable. Data are expressed as percentage of total
660 radioactivity recovered in total lipid and are means ± SD (n = 4).

661 **Table 3** Incorporation of [1-^{14}C]-labelled fatty acids into total lipid and lipid classes in FHM cells

	Substrate					
	[1-^{14}C]16:0	[1-^{14}C]18:1n-9	[1-^{14}C]18:2n-6	[1-^{14}C]18:3n-3	[1-^{14}C]20:5n-3	[1-^{14}C]22:6n-3
Total incorporation (pmol mg^{-1} protein)	315 ± 12^{a}	382 ± 37^{b}	432 ± 36^{b}	561 ± 13^{c}	430 ± 13^{b}	239 ± 24^{d}
Incorporation into lipid classes (% of total)						
- PtdCho	48.7 ± 3.5^{ab}	50.2 ± 3.0^{a}	43.8 ± 1.7^{b}	38.8 ± 0.4^{c}	31.8 ± 2.2^{d}	18.8 ± 0.3^{e}
- PtdEtn	16.2 ± 1.6^{a}	21.5 ± 0.5^{b}	18.2 ± 0.2^{a}	40.3 ± 0.8^{c}	52.9 ± 1.9^{d}	49.7 ± 0.7^{e}
- PtdSer	5.3 ± 0.1^{a}	6.1 ± 0.8^{ab}	6.8 ± 0.3^{b}	6.9 ± 0.3^{b}	7.0 ± 0.4^{b}	23.5 ± 0.7^{c}
- PtdIns	5.7 ± 0.2^{a}	8.3 ± 0.9^{b}	12.2 ± 1.9^{c}	2.7 ± 0.1^{d}	3.7 ± 0.2^{ad}	3.3 ± 0.2^{d}
- PtdOH/Ptd$_2$Gro	2.9 ± 1.4^{a}	2.0 ± 0.3^{a}	10.7 ± 0.3^{b}	7.1 ± 0.8^{c}	1.5 ± 0.1^{a}	1.5 ± 0.1^{a}
- CerPCho	13.4 ± 3.4^{a}	5.2 ± 3.0^{b}	2.9 ± 1.0^{bc}	1.0 ± 0.3^{bc}	0.5 ± 0.2^{c}	0.5 ± 0.1^{c}
- TN	7.7 ± 0.2^{a}	6.7 ± 0.2^{b}	5.4 ± 0.3^{c}	3.3 ± 0.0^{d}	2.7 ± 0.2^{e}	2.7 ± 0.1^{e}

662 Data are the mean ± SD (n = 4). Within each row, values sharing the same superscript are not significantly different at the P = 0.05 level. Abbreviations:
663 CerPCho, sphingomyelin; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdSer,
664 phosphatidylserine; Ptd$_2$Gro, cardiolipin; TN, total neutral lipids

665

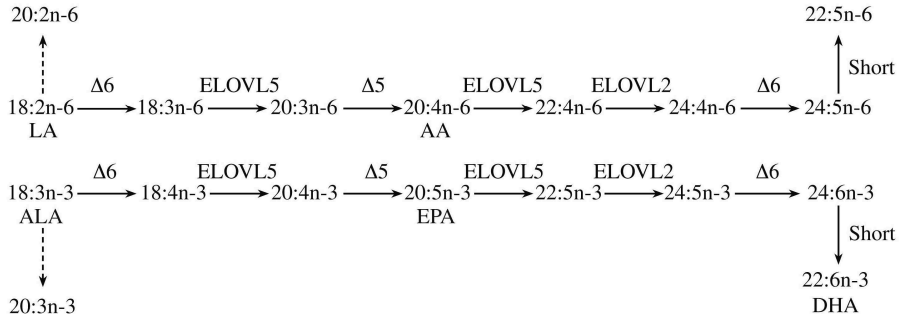

Fig. 1
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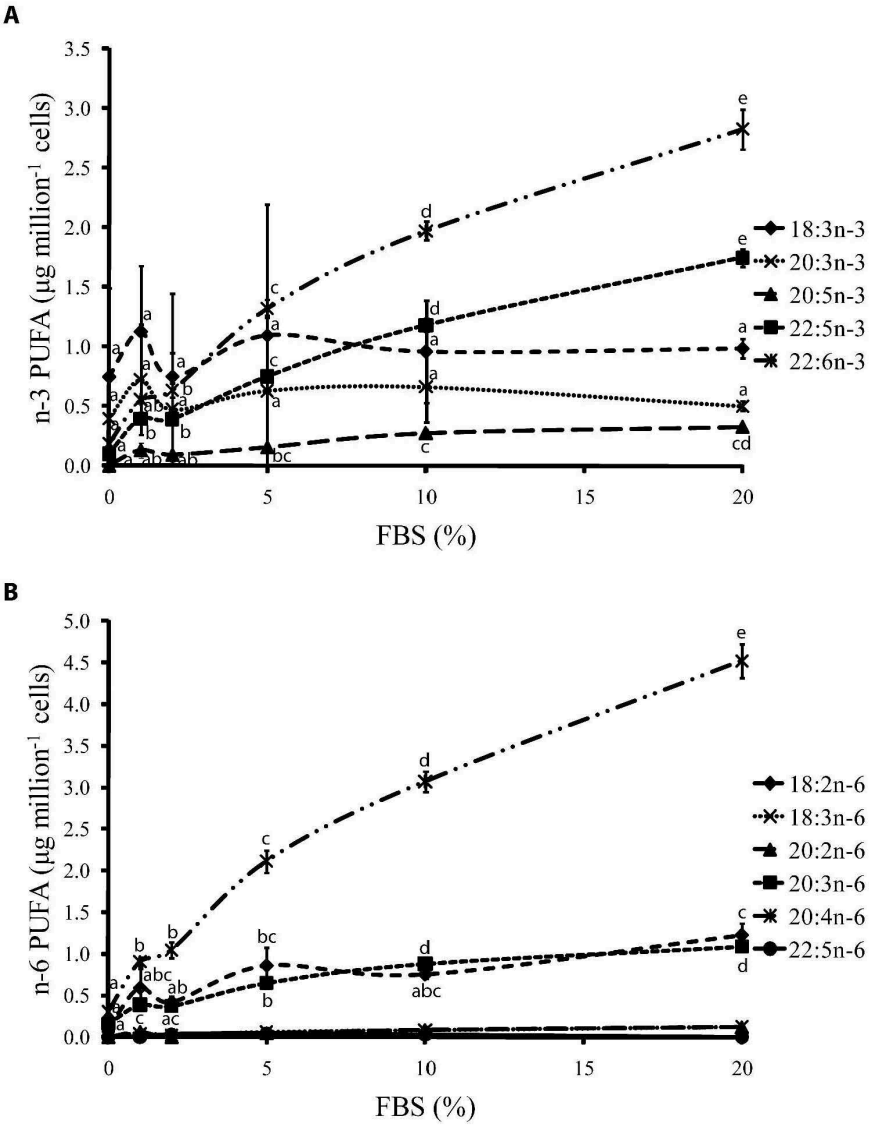
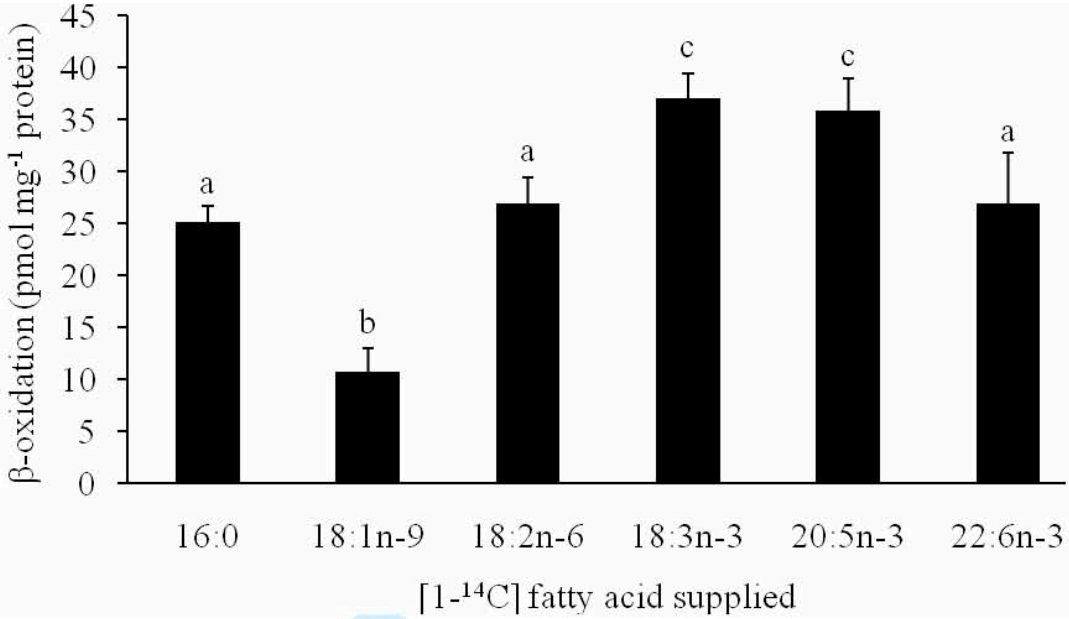


Fig. 2
194x245mm (600 x 600 DPI)



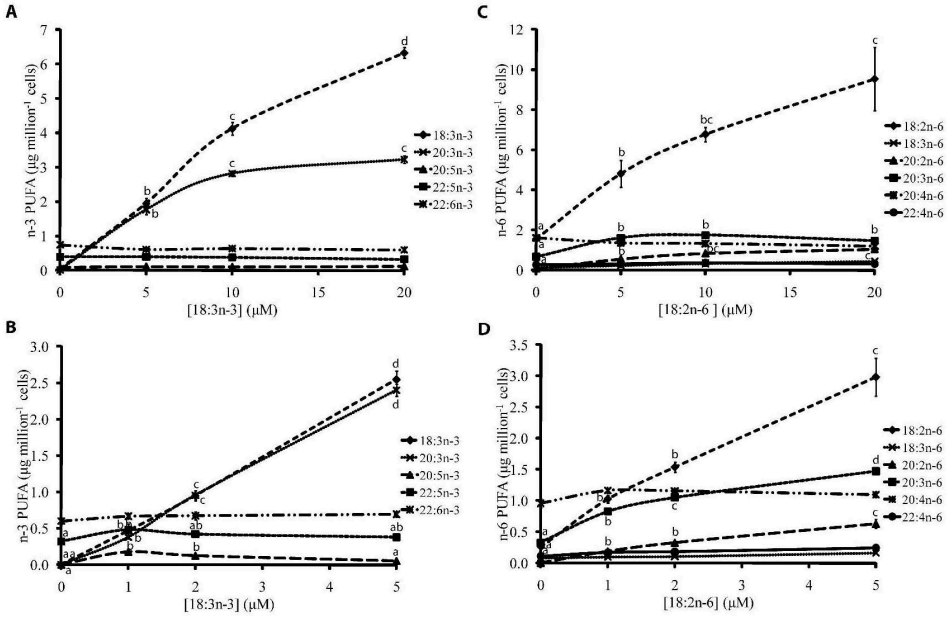


Fig. 4
213x139mm (600 x 600 DPI)

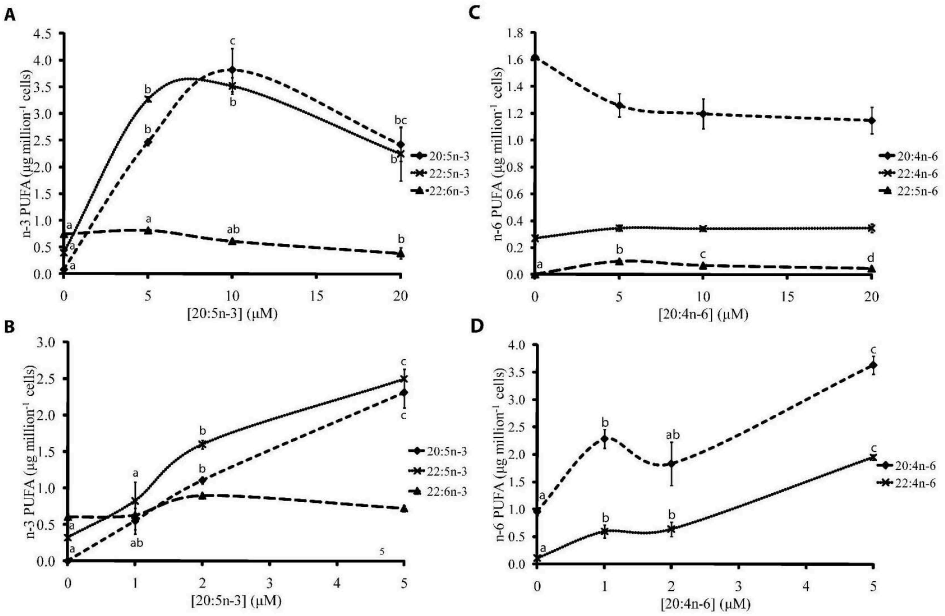


Fig. 5
212x138mm (600 x 600 DPI)

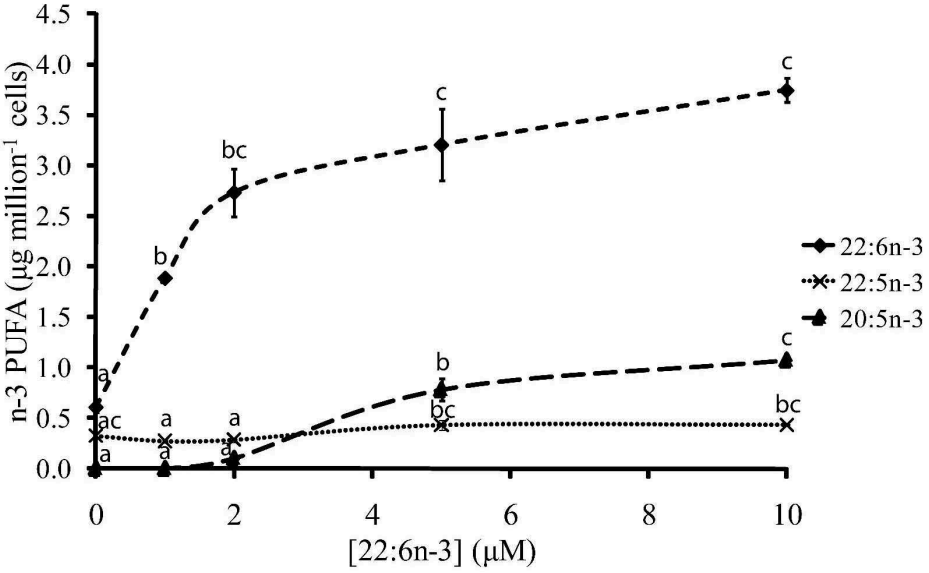
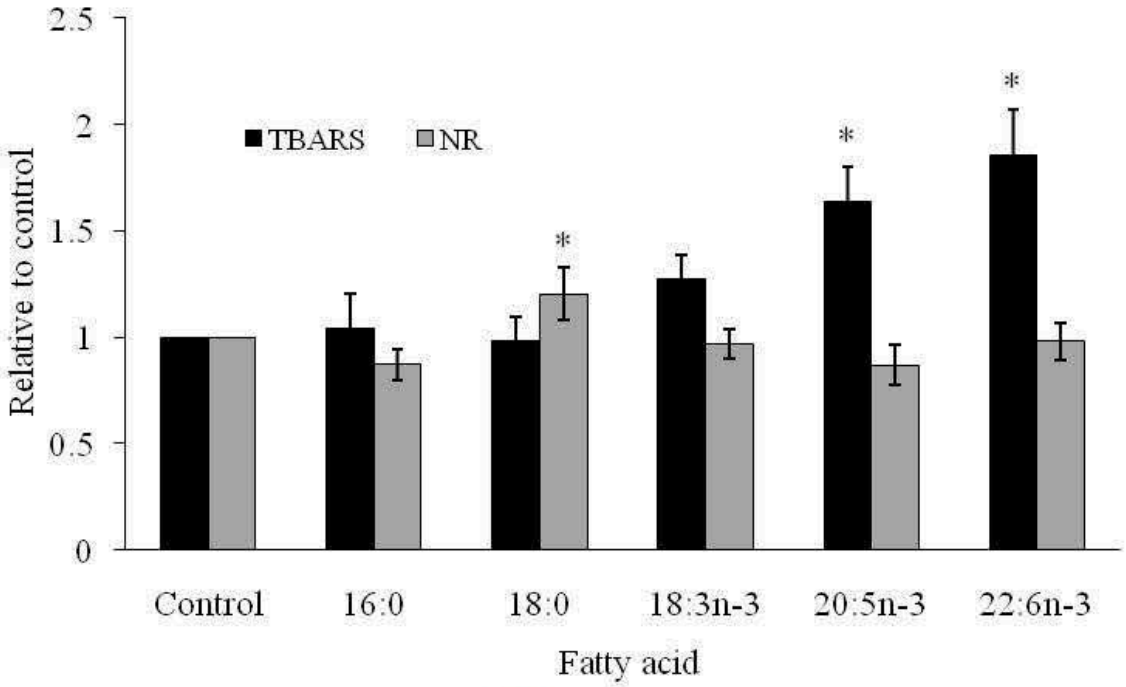


Fig. 6
204x125mm (600 x 600 DPI)


2.5
2
1.5
1
0.5
0
Relative to control
TBARS
NR
*
*
*
Control
16:0
18:0
18:3n-3
20:5n-3
22:6n-3
Fatty acid